JHR 91: 69–81 (2022)
doi: 10.3897/jhr.91.84139
https://jhr.pensoft.net

SHORT COMMUNICATION

JOURNAL OF Hymenoptera RESEARCH
A peer-reviewed open-access journal
The International Society of Hymenopterists

Detection of *Centistes* sp. (Hymenoptera, Braconidae) from intercepted *Diabrotica undecimpunctata* (Coleoptera, Chrysomelidae) using CO1 DNA barcodes and larval morphology

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Academic editor: Jose Fernandez-Triana | Received 22 March 2022 | Accepted 7 June 2022 | Published 30 June 2022

http://zoobank.org/842B0143-D48F-4642-8BD0-0D6A16FDAA99

Citation: Wilson CR, Cooke-McEwen C, Gilligan TM, Tembrock LR (2022) Detection of *Centistes* sp. (Hymenoptera, Braconidae) from intercepted *Diabrotica undecimpunctata* (Coleoptera, Chrysomelidae) using CO1 DNA barcodes and larval morphology. Journal of Hymenoptera Research 91: 69–81. https://doi.org/10.3897/jhr.91.84139

Abstract

Globalized trade has resulted in the incidental translocation of numerous insect species, some of which have become invasive in their expanded ranges. While rigorous inspection programs are a regular part of commodity importation, rarely if ever are the internal contents of intercepted insects examined. As part of a genetic diversity project on intercepted *Diabrotica undecimpunctata* beetles, we detected CO1 DNA that closely matched sequences from *Centistes* parasitoid wasps in 9% of our samples. The presence of internal parasitoids was confirmed through dissections and imaging, wherein the samples were morphologically consistent with *Centistes* larvae. Such a discovery suggests that insect translocation as part of trade can be more diverse than initially thought. The case of *Centistes* in imported *Diabrotica* may present a positive benefit specifically to agroecosystems through the biological control of pest beetles like *Diabrotica*. However, drawbacks from such introductions include off-target parasitism of non-pest beetles and resultant impacts to insect populations in undisturbed ecosystems. Thus, examination of intercepted insects beyond the initial species identification is warranted to better understand the potential impacts of human mediated insect translocations. Methods employing high-throughput sequencing and metabarcoding are well suited for such broad-scale identification projects where *Diabrotica* would be an excellent candidate for this work.

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Keywords
Agricultural trade, biological control, mitochondrial DNA, molecular identification, parasitoids, spotted cucumber beetle

Introduction

In 2021, the value of United States (US) agricultural imports exceeded $14.6 billion (USDA 2021). Inherent in the trade of agricultural commodities is the incidental importation of insects, some of which have the potential to establish as important pests of domestic crops (Work et al. 2005; Hulme 2009; Paini et al. 2016). *Diabrotica undecimpunctata* Mannerheim (Coleoptera: Chrysomelidae) is a beetle pest species known to cause economic losses to crops such as cucumber, maize, and soybean in its native range of North America (Hirsh and Barbercheck 1996; EFSA 2020). The species *D. undecimpunctata* is frequently intercepted on agricultural commodities being brought from Mexico for import into the U.S. For example, from a single high-volume port-of-entry between Mexico and the U.S., 206 *D. undecimpunctata* individuals were identified from January 2018 to December 2020 (USDA internal interception database - Agricultural Risk Management System - ARM). Beyond the initial insect identification, rarely if ever are associated species identified from these beetles (or from any intercepted insect for that matter) even though *D. undecimpunctata* is a vector of several destructive plant pathogens (Milbrath et al. 1975; Yao et al. 1996) and is known to be susceptible to parasitoid wasps (Toepfer et al. 2008).

Given the lack of studies regarding parasitoid translocation, we examined recent *D. undecimpunctata* interceptions to determine if parasitoids could be found in intercepted beetles and if so in what abundance. To do this CO1 barcodes attained from 109 intercepted *Diabrotica* beetles were scanned for evidence of parasitoid wasp DNA. Ten individuals were found to have detectable hymenopteran DNA through comparisons to public databases and further dissected to confirm the presence of parasitoid larvae through morphological examination. Considering the parasitoid fauna of intercepted insects provides a rich data set to characterize agricultural biodiversity more comprehensively as well as assess threats or benefits from such organisms to domestic agriculture. While our study employed a relatively simple approach it clearly showed that *Diabrotica* parasitoids are being translocated through trade in agricultural commodities. Additional work should be conducted to better understand how such parasitoid translocation might affect native beetle populations including the reduction of pest species from agroecosystems.

Materials and methods

DNA extraction, PCR, and sequencing

In this study, DNA was extracted from 109 intercepted (from agricultural commodities), ethanol preserved, whole individual beetle specimens using a (nondestructive)

Lucigen MasterPure DNA extraction kit (Lucigen Corp., Middleton, WI, USA) with modifications (Zink et al. 2019). Universal primers Nancy (Simon et al. 1994) and S1718 (Jordal et al. 2004) were used to optimize compatibility with previously generated data sets (Swigoňová and Kjer 2004; Eben and Espinosa de Los Monteros 2013). The following PCR protocol was performed in 50 µL volumes containing 32.75 µL of ddH2O, 5.0 µL of 10× Ex TAQ buffer, 4.0 µL of dNTP mixture at 2.5 mM, 1.0 µL of S1718 primer at 0.01 mM, 1.0 µL Nancy primer at 0.01 mM, 2.5 µL of MgCl2 at 25 mM, 2.5 µL BSA at 20 mg/mL, 0.25 µL of TaKaRa Ex Taq HS (Takara Bio Inc., Shiga Japan) at 5 U/µL, and 1 µL of DNA template or water for no tissue controls. All PCR was carried out on a BioRad C1000 Touch Thermocycler (BioRad Laboratories, Inc., Hercules, CA, USA) using the following program: 1) 3 mins at 94 °C, 2) 20 secs at 94 °C, 3) 20 secs at 52 °C, 4) 30 secs at 72 °C, 5) cycles 2–4 repeated 39 times, and 6) a final extension of 5 minutes at 72 °C. A lid temperature of 105 °C was maintained throughout all cycles. Cross contamination was prevented by sanitizing all equipment and work areas on regular basis. Success of PCRs were confirmed on 1% agarose gels using ethidium bromide to visualize DNA in UV light. The reactions that produced visible bands were purified using a Qiagen QIAquick PCR purification kit following the manufactures instructions (Qiagen Inc., Düsseldorf, Germany). Purified samples were sequenced at the University of Chicago Cancer Research Center DNA Sequencing Facility using an Applied Biosystems 3730XL DNA sequencer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were contiged after manually trimming poor quality base calls from the 5' and 3' ends of the sequences. Contiged sequences were converted into consensus sequences and used in all subsequent analyses. Sequences that could not be contiged due to poor read quality or indecipherable 'double reads' were excluded from further analyses.

CO1 sequence searches and phylogenetic analyses

The molecular identification of samples using CO1 barcodes generated in this study employed several different strategies. First each CO1 sequence was queried against the NCBI nucleotide database using BLASTn (Altschul et al. 1990) and to the public data available in the Barcode of Life Database (Ratnasingham and Hebert 2007) (Table 1). In both searches, default settings were used. Samples that returned high confidence matches to non-coleopteran species were further assessed together using three different phylogenetic approaches. Specimen codes (PfD numbers) and their corresponding GenBank accession numbers are listed in Table 1. In order to generate a comparative set of sequences, the longest sequence (PfD 027, 484bp) from the ten non-coleopteran samples was BLASTn searched against the complete GenBank database. From this search, the 250 sequences with the lowest e-values were aligned to the 10 non-coleopteran CO1 sequences from this study using MAFFT v 7.450 (Katoh et al. 2002; Katoh and Standley 2013). The alignment (Suppl. material 1) was used to generate a NJ (Neighbor Joining) distance tree (Suppl. material 1: Fig. S1) using the Tamura-Nei distance model (Tamura and Nei 1993). From this tree, a subset of samples was selected that clustered with

Table 1. Results of database searches using CO1 barcode sequences from intercepted *Diabrotica* beetles as queries. Parasitoid determinations were based on database matches and examination of morphological characters from dissections when possible. Agricultural commodity refers to the plant material on which the *Diabrotica* beetles were found, all interceptions were made from shipments originating from Mexico with destinations in the US. The last two columns are the best hits from searches to the BOLD and GenBank (using BLASTn) databases respectively.

Lab ID	Parasitoid Determination	Agricultural Commodity	GenBank Accession	Barcode of Life Database (% similarity)	BLASTn (e-value for best hit)
PfD 002	Braconidae *Centistes* sp.	Romaine Lettuce	ON713979	Hymenoptera Braconidae *Centistes* (92.23)	*Centistes* sp. (2e-123)
PfD 027	Braconidae *Centistes* sp.	Romaine Lettuce	ON713980	Hymenoptera Braconidae *Centistes* (92.74)	Hymenoptera sp. (4e-165)
PfD 065	Braconidae *Centistes* sp.	Romaine Lettuce	ON713981	Hymenoptera Braconidae *Centistes* (92.06)	*Centistes* sp. (3e-121)
PfD 074	Braconidae *Centistes* sp.	Lettuce	ON713982	Hymenoptera Braconidae *Centistes* (92.05)	Hymenoptera sp. (8e-127)
PfD 076	Braconidae *Centistes* sp.	Celery	ON713983	Hymenoptera Braconidae *Centistes* (91.84)	*Centistes* sp. (1e-115)
PfD 080	Braconidae *Centistes* sp.	Romaine Lettuce	ON713984	Hymenoptera Braconidae *Centistes* (92.96)	Hymenoptera sp. (1e-150)
PfD 088	Braconidae *Centistes* sp.	Lettuce	ON713985	Hymenoptera Braconidae *Centistes* (92.33)	Hymenoptera sp. (8e-127)
PfD 091	Braconidae *Centistes* sp.	N/A	ON713986	Hymenoptera Braconidae *Centistes* (90.13)	Hymenoptera sp. (8e-127)
PfD 096	Braconidae *Centistes* sp.	N/A	ON713987	Hymenoptera Braconidae *Centistes* (91.76)	Hymenoptera sp. (2e-147)
PfD 105	Braconidae *Centistes* sp.	N/A	ON713988	Hymenoptera Braconidae *Centistes* (91.67)	Hymenoptera sp. (4e-109)

the ten non-coleopteran samples in the NJ tree, and a group that did not cluster with this set to be treated as an outgroup. The selected samples were realigned and trimmed to eliminate all non-overlapping loci and realigned again using MAFFT. Using this 42 sample by 336 nucleotide matrix (Suppl. material 1), three different tree-building approaches were used to test the robustness of the relationships inferred in the first NJ pass. The methods used were: 1) NJ using the Tamura-Nei distance model and 1,000 jack knife replicates to assess branch support 2) Maximum likelihood using PhyML 3.3.2 (Guindon et al. 2010) using a GTR nucleotide substitution model, fixed proportion of invariable sites, an estimated gamma distribution parameter, and 1,000 bootstrap pseudoreplicates to assess branch support, and 3) Bayesian inference (BI) using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) with a GTR+I+G substitution and rate variation model, 4 gamma categories, 4 heated chains, a chain length of 1,100,000, a subsampling frequency of 200, a burn-in of 100,000; and an unconstrained branch length prior. An additional MrBayes run was conducted using the JC69 substitution model (all other settings same as above) to assess differences in tree topology and branch support when applying the simplest substitution model. Running both nucleotide substitution models was employed given that it is a more exhaustive approach than model testing in assessing differences in tree topology and support under different models.

In the NJ and BI methods *Microctonus aethiopoides* JN980133.1 was used as an outgroup given its position outside of *Centistes* yet within Euphorinae. Trees were visualized in Figtree v 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Beetle dissection and parasitoid larval imaging

Beetle DNA samples that produced a CO1 sequence that were non-coleopteran were further examined through dissection to determine if parasitoid larvae were present. Beetles were dissected after soaking in 10% KOH for approximately 60 minutes. The elytra and wings were removed, and the abdomen and thorax were cut open laterally using small Vannas spring scissors (Fine Science Tools, Foster City, CA). The contents of the body were searched for parasitoids, which were transferred to 100% ethanol prior to slide mounting using Euparal. Slide-mounted parasitoids were imaged using a Nikon Eclipse 80i compound phase contrast/DIC microscope equipped with a Nikon DS-Fi2 digital camera (Nikon Instruments, Inc., Melville, NY). Photos were taken with Nikon Elements v 4.60 imaging software and edited in Adobe Photoshop.

Results

From a sample set of 109 *D. undecimpunctata*, 10 produced sequences that resolved with CO1 sequences from the hymenopteran genus *Centistes* Haliday (all references to this genus are *sensu* Stigenburg et al. 2015 unless noted otherwise) as determined from searches to large public databases (Table 1), and tree-based analyses (Fig. 1). Queries to the large public databases GenBank and BOLD found no species-level matches due to incomplete sampling in these databases (Table 1). In all tree-based methods of resolution the ten CO1 sequences derived from *D. undecimpunctata* clustered together with high branch support. In the NJ and ML trees *Centistes* sp. KJ591434 (Suppl. material 1: Figs S1–S3) resolved closest to the clade of parasitoids from *D. undecimpunctata* but with low support. In the two BI runs (Suppl. material 1: Figs S4, S5) the parasitoids from *D. undecimpunctata* resolved in a clade with *Centistes* sp. KJ591434, *Centistes* sp. KJ591429, and *Centistes* sp. KJ591430 with high PP support (GTR 0.95 and JC69 0.96). Within the clade containing the ten parasitoids from *D. undecimpunctata*, several different topologies were resolved between the different methods but in all cases PfD 027, 065, 074, 080, 088, and 105 were resolved in a clade with high support (JK 76, BS 70, and PP from the GTR model 0.99) and the samples 002, 076, 091, and 096 in a grade (ML and BI with GTR) or sister relationship position (NJ and BI with JC69) to that clade.

Beetles that produced non-coleopteran CO1 barcodes, were dissected and carefully examined for the presence of larval parasitoids. In four of the ten *Diabrotica* beetles dissected recognizable parasitoid larvae were recovered (Fig. 2). In the remaining six beetles dark, unrecognizable masses were recovered similar to that found in PfD 076 (Fig. 2D) but without the presence of identifiable morphological characteristics. These dense unrecognizable masses may represent diapausing larvae, a life cycle strategy that

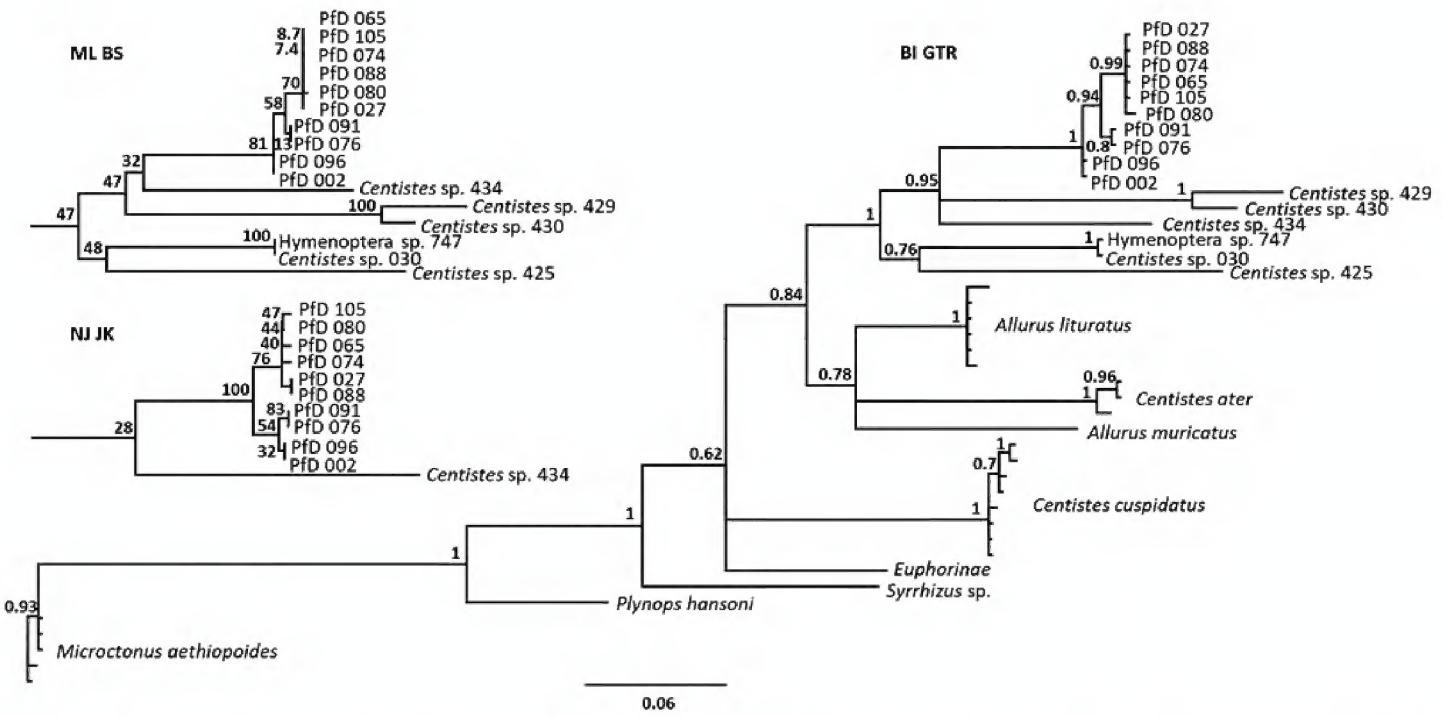

Figure 1. Phylogenetic analyses using CO1 sequences from parasitoids in *D. undecimpunctata* aligned with the most similar sequences from NCBI. Complete tree is BI using the GTR nucleotide substitution model with PP branch support, subtrees show results from the NJ (JK support) and ML (BS support) methods to highlight the similarities in resolution between methods in relation to the samples generated in this study. All analyses used the same MAFFT alignment (Suppl. material 1). Complete trees for all methods are included in Suppl. material 1: Figs S1–S5. Numbers after species names refer to the last three digits of the GenBank accessions, all other numbers refer to internal lab accession numbers which can be cross referenced in Table 1.

is known to occur in *Centistes* and related braconids (Zijp and Blommers 2002; Barker 2013). Images taken for PfD 002, 009, and 105 have clearly identifiable sclerotized head capsules which is consistent with the Braconidae (Stehr 1987). These specimens also had smooth, toothless mandibular blades (Fig. 2A, B, C, E, F) which is consistent with larval traits of species from the subfamily Euphorinae and the genus *Centistes* (Čapek 1970; Stehr 1987; Quicke 2015).

Discussion

During the course of a project to characterize the genetic diversity of *D. undecimpunctata* beetles using CO1 barcodes we found ten specimens contained sequences that were consistent with the parasitoid hymenopteran genus *Centistes* (Table 1; Fig. 1). Upon dissection of these beetle specimens (Fig. 2) four contained a single larva that was morphologically consistent with Braconidae larvae and in large part morphologically consistent with descriptions of *Centistes* larvae (Čapek 1970; Stehr 1987; Quicke 2015). The remaining six dissections contained dark non-descript masses that we suspect were diapausing *Centistes* larvae. The mandibles in *Centistes* are generally short (not crossing medially) and wedge-shaped (van Achterberg 1976; Čapek 1970; Stehr 1987; Quicke 2015) as opposed to the longer, more sickle-shaped mandibles observed

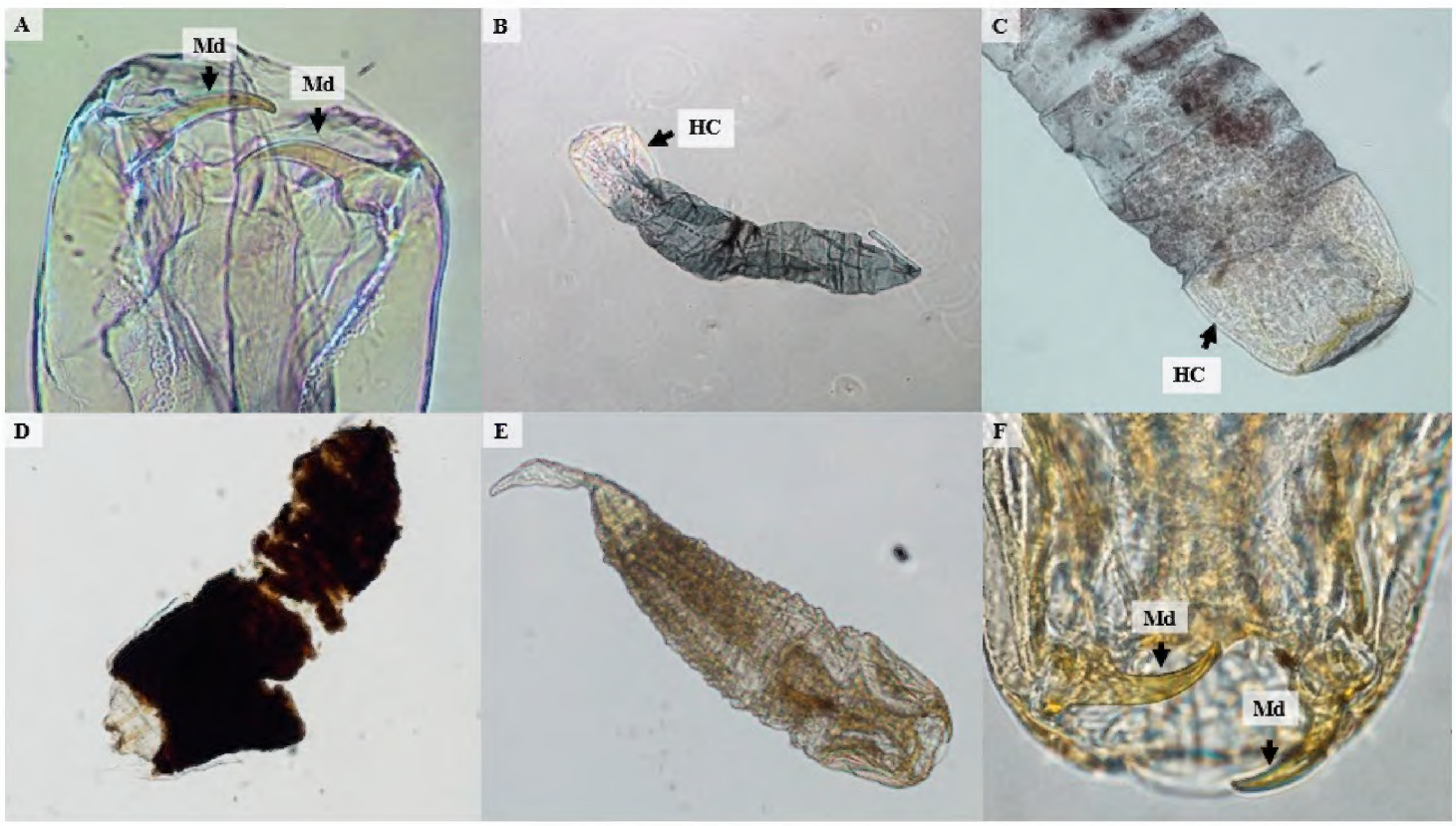

Figure 2. *Centistes* larvae dissected from *Diabrotica* beetles that produced CO1 sequences with matches to *Centistes* (see Table 1). Arrows with **Md** indicate mandibles, **HC** indicate sclerotized head capsules consistent with Braconidae and in part with *Centistes*. Note the lack of teeth on the mandibular blades separating Euphorinae from Meteorideinae, Helconinae, and Agathidinae which all have toothed blades. Panels **A, B** are PfD 002 **C** PfD 009 **D** PfD 076, and **E, F** PfD 105.

in our specimens. Because DNA evidence places our specimens within *Centistes* the differences seen in mandible size may represent derived traits not previously noted for *Centistes*. Such a gap in knowledge would not be unexpected given that the variability of cephalic structures in North American *Centistes* larvae has probably not been fully documented (Forbes et al. 2018) and thus the range of mandible morphology is not known. In addition to morphology, the fact that our specimens were koinobiont endoparasitoids from chrysomelid beetles further confirms their membership in Euphorinae and Centistini given that this combination of traits are essentially unique to these groups (van Achterberg 1976; Quicke and van Achterberg 1990; Quicke 2015).

The results of the Sanger sequencing data in this study are somewhat unexpected in that it is assumed the number of beetle reads would far exceed the number of parasitoid reads based on the size of the two organisms. Several technical and biological factors may help explain why parasitoid Sanger reads were recorded in greater number than beetle reads. First, it should be noted that the electropherograms for samples that produced *Centistes* sequences also contained higher levels of background peaks suggesting that both beetle and parasitoid barcodes were being amplified and sequenced (Suppl. material 1: Fig. S6). This is also evident at the level of base calling where double peaks resulting from double reads lead to more ambiguous base calls in the parasitoid sequences than the beetle only reads. In fact, equimolar amounts of parasitoid and beetle DNA may explain why some amplicons returned unreadable Sanger electropherograms and could not be contiged (Crossely et al. 2020). Several technical issues

regarding PCR efficiency may also have influenced our results (Booth et al. 2010). A factor often affecting PCR efficiency is primer template mismatches (Cha and Thilly 1992; Stadhouders et al. 2010). Yet from the available data in GenBank for *Diabrotica* and *Centistes* the S1718 and Nancy primers would be expected to perform less well in amplifying *Centistes* CO1 given the greater number of mismatches. The amplicon length and GC content (known factors effecting PCR efficiency: Qu et al. 2009; Mallona et al. 2011) are similar between *Centistes* and *Diabrotica* and do not provide an obvious explanation for the recovery of a greater quantity of *Centistes* reads.

Given the lack of an obvious technical explanation, biological attributes should be considered to help clarify how CO1 reads from the nearly microscopic *Centistes* larvae could saturate those from the comparatively massive host. The differences in life stage between larval *Centistes* and adult *Diabrotica* may underlie differences in mtDNA copy number as has been shown in different life stages of female *Drosophila* (Salminen et al. 2017). Transfer of genes from the mitochondria to the nucleus, which is known for CO1 in several parasitoid wasps (Viljakainen et al. 2010; Yan et al. 2019), might also explain increases in CO1 read number between parasitoid and host by increasing the total number of CO1 copies per cell. Lastly a wide variety of parasitoid induced alterations of host metabolism have been described (Mrinalini et al. 2015; Wang et al. 2021) and may in turn affect the copy number of host mitochondria.

The presence of clearly identifiable braconid larvae in at least three of the beetles that produced CO1 sequences matching to *Centistes* provides unequivocal confirmation that these beetles were parasitized. What is less clear is the exact identity of the parasitoid given the limited number of *Centistes* CO1 sequences available in public repositories and the difficulty in making species level identifications from larvae. Despite this lack of available sequence data, the species *C. diabroticae* and *C. gasseni* have been repeatedly described from the literature to feed on several adult species of *Diabrotica* and related *Acalymma* (Toepfer et al. 2008; Smyth and Hoffmann 2010). Of these two species, *C. gasseni* is known from rearing experiments to be able to parasitize *D. undecimpunctata* but is not known from Mexico, and *C. diabroticae* is known from Mexico but has not been reported to parasitize *D. undecimpunctata* (Shaw 1995; Toepfer et al. 2008). While recent molecular phylogenetic work has been conducted on *Centistes* (Stigenburg et al. 2015), *C. diabroticae* and *C. gasseni* were not among those sampled and therefore sequences for these species are currently not available in BOLD or GenBank. While it is probable that the larvae we found inside intercepted *D. undecimpunctata* were *C. diabroticae* or *C. gasseni*, because of the large number of new *Centistes* species that are regularly discovered in the Americas (Shaw 1995; Aguirre et al. 2017) it is possible that our specimens from Mexico could represent a previously undescribed taxon. Given this, further studies are needed to clarify what *Centistes* species are parasitizing *D. undecimpunctata* in Mexico and being brought north through agricultural trade, if they are part of a biocontrol program or the result of natural parasitism, and whether these *Centistes* species could be employed in the U.S. to control pest *Diabrotica*.

It has long been known that insects harbor a broad array of species in and on their bodies including parasitoids, arachnids, fungi, bacteria, and viruses (Purcell 1982; Knell

and Webberley 2004; Wielkopolan et al. 2021). In efforts to more comprehensively understand the effects of insect translocation on agroecosystems, improved sampling of insect bodies should be conducted. For such work, a number of sequencing and molecular assay approaches are available that can identify different species simultaneously (e.g., Skelton et al. 2019; Zink et al. 2019; Verdasca et al. 2021). Given our results presented here and what is known about the microbes that *D. undecimpunctata* can vector, this beetle stands as a superb candidate for such sampling projects in the future.

Acknowledgements

We thank Frida A. Zink and Alicia E. Timm for their helpful insights on numerous technical aspects of this study and Charlotte Aldebron for discussions on beetle and parasitoid biology. We thank Anthony Boughton, Tyler Hedlund, Garrett Hughes, and Alexander S. Konstantinov for their ongoing work to collect, identify, and preserve *Diabrotica* beetles for use in this and similar studies. Funding was provided in part through USDA cooperative agreement AP19PPQS&T00C136 to Luke R. Tembrock at Colorado State University. The authors declare no competing interests.

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Supplementary material 1

DNA alignments and figures

Authors: Luke R. Tembrock, Christina R. Wilson

Data type: Docx file.

Explanation note: DNA alignments, Sanger electropherograms, and phylogenetic trees.

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